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VIROLOGY 172, 180–188 (1989)

Antigenic and Molecular Characterization of Subtype H13 Hemagglutinin of Influenza Virus

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Received February 14, 1989; accepted May 31, 1989

Influenza A viruses with subtype H13 hemagglutinin display an unusual host range. Although common in shorebirds, they are very rare or absent in wild ducks; additionally, H13 viruses have been isolated from a whale. To study the molecular basis for this host range, we have determined the complete nucleotide sequences of the hemagglutinin genes of three H13 influenza viruses from different species or geographical areas: A/gull/Maryland/77, A/gull/Astrachan (USSR)/84, and A/pilot whale/Maine/84. Based on the deduced amino acid sequences, H13 hemagglutinin shares the basic structure of other type A hemagglutinin subtypes such as H3, but has clearly diverged from other completely sequenced subtypes. Unique features of H13 hemagglutinin include the occurrence, near the receptor binding pocket, of residues Arg/Lys-227 and Trp-229 (H3 numbering); the significance of these are unknown. The sequence of the HA1–HA2 cleavage site resembles those of avirulent avian influenza viruses. The whale H13 hemagglutinin is similar to those from gulls, supporting the hypothesis that influenza viruses from avian sources can enter marine mammal populations but are probably not permanently maintained there. Antigenic analysis using a panel of monoclonal antibodies suggests that, like other subtypes, H13 viruses are heterogeneous, with different antigenic variants predominating in the eastern versus the western hemispheres.

INTRODUCTION

Influenza A viruses encode two surface glycoproteins, hemagglutinin (HA) and neuraminidase. The HA molecule mediates virus entry into cells through receptor binding and membrane fusion, and is the primary target of the host antiviral antibody response (reviewed in Wiley and Skehel, 1987). Thirteen subtypes of HA, which are not serologically cross-reactive, have been identified which distinguish influenza A viruses. Only a few of these are found in viruses commonly infecting mammals; e.g., H1, H2, and H3 in humans. However, wild migrating waterfowl including ducks and shorebirds are carriers of viruses of all the 13 HA subtypes, and sometimes transmit them to other species (reviewed in Webster and Kawaoka, 1988). Outbreaks of H4 and H7 influenza in seals, and H10 influenza in mink, have been attributed to viruses bearing avian-like HA (Feldmann *et al.*, 1988, Hinshaw *et al.*, 1984, Webster *et al.*, 1981). In humans, the emergence of new pandemic influenza viruses is largely due to the introduction of a virus with a novel HA subtype into an immunologically naive population. For example, the H3N2 influenza viruses currently circulating in humans are believed to be directly descended from a ca. 1968 reassortment event between a human H2N2 virus and a nonhuman H3 virus related to the Duck/Ukraine/63 virus (Laver and Webster, 1973). Additionally, functional as well as antigenic characteristics of different HA subtypes are determinants of viral pathogenicity. The presence of multiple basic amino acids at the HA1–HA2 cleavage site confers sensitivity to cleavage by a wider variety of cellular proteinases and is characteristic of the highly virulent H5 and H7 influenza viruses presumed to be maintained in wild waterfowl and sometimes transmitted to domestic poultry (Kawaoka *et al.*, 1987; Bosch *et al.*, 1981).

The H13 HA was the last HA subtype to have been discovered (Hinshaw *et al.*, 1982). H13 viruses appear to be unique in their host range: while they have been frequently isolated from shorebirds such as gulls in both the eastern and western hemispheres (Hinshaw *et al.*, 1982; Kawaoka *et al.*, 1988), no H13 viruses were detected among over 4300 influenza viruses isolated from ducks in the United States, Canada, or Hong Kong over a 12-year period (Hinshaw *et al.*, 1980, 1985; R. G. Webster, unpublished data). H13 viruses have also been isolated from the lung and hilar node of a diseased pilot whale (Hinshaw *et al.*, 1986). Unlike other avian influenza viruses, most H13 viruses tested do not replicate in the intestinal tracts of ducks experimentally infected by the oral or intratracheal routes. H13 viruses do replicate in the intestinal tract of ferrets (Hinshaw *et al.*, 1982, 1986).

These results have led us to examine the molecular features of the H13 HA that might be responsible for

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Sequence Data from this article have been deposited with the EMBL/GenBank Data Libraries under Accession No. J04370.

0042-6822/89 $3.00

the unusual host range of H13 viruses. Here we report the complete nucleotide sequence and deduced amino acid sequence of H13 HAs from gull and whale viruses. Additionally we assess the antigenic variability and evolutionary pattern of H13 HAs from two geographical sources of viruses.

MATERIALS AND METHODS

Viruses

Subtype H13 influenza viruses from the USA were obtained from the repository at St. Jude Children's Research Hospital. Viruses from the USSR were from the D. I. Ivanovsky Institute. The viruses used for sequencing included influenza A/ring-billed gull/Maryland/704/77 (H13N6) (MD), which serves as the prototype H13 virus (Hinshaw *et al.*, 1982), A/pilot whale/Maine/328HN/84 (H13N9) (WH) (Hinshaw *et al.*, 1986), and A/black-headed gull/Astrachan (USSR)/227/84 (H13N6) (AS). These viruses were grown in 11-day-old embryonated chicken eggs and purified by sucrose gradient centrifugation, and their RNA was isolated as described (Bean *et al.*, 1980).

Nucleotide sequence determination

Direct RNA sequencing was done by the dideoxynucleotide chain termination method (Sanger *et al.*, 1977; Air, 1979) using the purified viral RNA as template, and reverse transcriptase. Primers for reverse transcription were oligodeoxynucleotides of 12–18 bases, which were synthesized in an Applied Biosystems 380A DNA synthesizer. Sequencing reaction products were labeled using [^{35}S]dATP and separated on 7% polyacrylamide/7 *M* urea buffer gradient gels (Biggin *et al.*, 1983).

cDNA clones of the HA genes of these viruses were prepared as described (Kawaoka *et al.*, 1987) and were used to determine the sequences of the 5′ termini of these genes and to resolve ambiguities in the RNA-derived sequences. Plasmid sequencing was done using Sequenase (United States Biochemical Corp.) by the method of Tabor and Richardson (1987).

Monoclonal antibodies

Monoclonal antibodies to the hemagglutinin of AS virus were prepared according to the method of Kohler and Milstein (1976) as previously described (Kida *et al.*, 1982). Balb/c mice were immunized with two intraperitoneal injections of AS virus, 2 weeks apart. Each dose consisted of 5000 HA units (HAU) of whole virus and 5000 HAU of ether-disrupted virus. The hybridoma lines obtained were grown as ascites tumors in Balb/c mice. Ascitic fluids were screened for reactivity against AS HA by enzyme-linked immunosorbent assay, using detergent-disrupted AS virus as the antigen as described (Kida *et al.*, 1982) and by hemagglutination-inhibition (HI) tests. HI tests were performed as described (Palmer, 1975) using 4 HAU of each virus and 0.5% chicken erythrocytes.

RESULTS

Nucleotide and amino acid sequences of H13 HA genes

The sequence of the 5′ end (bases 1–311) of the HA of the MD virus has been previously reported (Hinshaw *et al.*, 1982). Based on this data, primers were designed to extend this sequence to the 3′ end of the gene by direct viral RNA sequencing. Also, a primer was designed matching bases 9–20 of the MD HA, which partly overlaps the universal influenza A 5′ RNA terminus and partly is unique to the HA gene in a relatively conserved region. This primer was used to begin sequencing of the WH and AS HA genes. The 3 HAs were sufficiently different that most primers proved to be useful with only 1 or 2 of the HA strains.

The complete nucleotide sequences of the 3 H13 HA genes are shown in Fig. 1. The MD and WH HA genes are each 1768 bases in length, with single open reading frames encoding unprocessed polypeptides of 566 amino acids. By comparison the AS H13 HA gene is 1765 bases in length, and encodes a 565 amino acid polypeptide. The size difference between the AS HA and the others is traceable to the region between bases 500 and 507 (MD sequence) where AACACG is replaced by CGA and thus Asn–Thr by Arg.

The degrees of nucleotide sequence homology among these viruses are shown in Table 1. The gull HAs, MD and AS, were not closely related (78% homology). The WH HA, while to an extent intermediate among the others, was more similar to AS HA (85% homology); about $\frac{2}{3}$ of WH differences from MD HA were shared with AS HA.

The deduced amino acid sequences of the encoded HA polypeptides are shown in Fig. 2, and their homologies in Table 1. All three HAs were relatively similar in HA2, whereas the MD and AS sequences were distinct in HA1. At the amino acid level, the WH HA more closely resembled an intermediate between the two gull HAs: among the 60 positions in HA1 which were not common among the 3 HAs, the WH HA shared 26 residues with MD HA and 23 others with AS HA, whereas AS HA shared only 5 with MD HA.

By analogy with H1, H2, H5, and H7 (Air, 1981; Kawaoka *et al.*, 1984) we have placed the amino terminus of HA1 at Asp, 19 residues from the beginning of the sequence, with the preceding 18 amino acids consti-

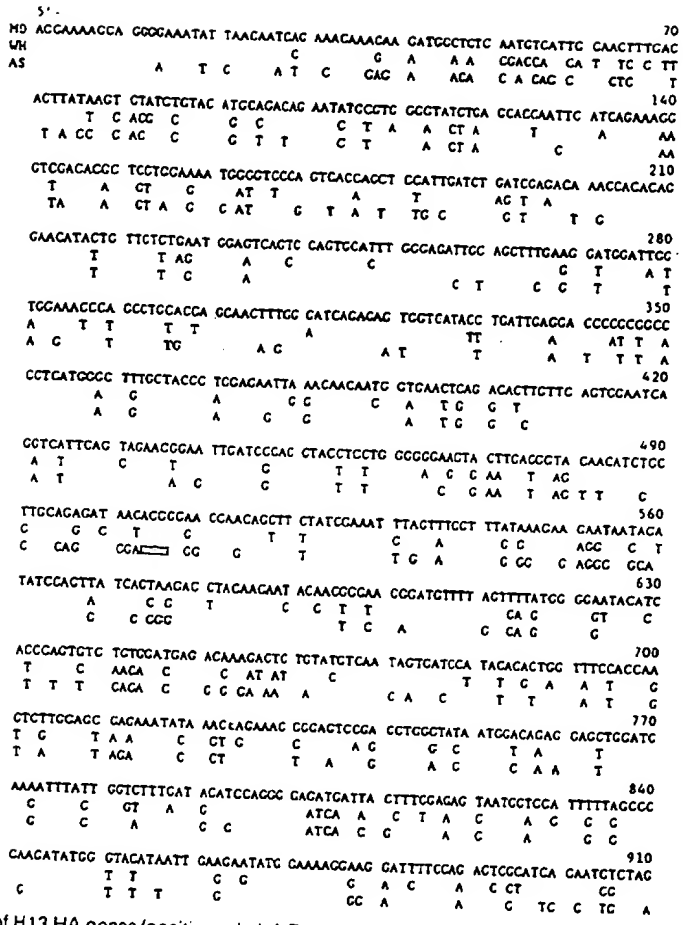

FIG. 1. Nucleotide sequences of H13 HA genes (positive polarity). The MD HA sequence is shown in entirety on the top line. Bases in the WH and AS HA sequences which differ from those in MD HA are shown underneath. The closed boxes in the AS HA sequence at positions 504–506 indicate a region where 3 bases are deleted relative to the MD sequence. The positioning of the boxes between bases 501 and 506 is arbitrary.

tuting the hydrophobic signal sequence for membrane insertion. In Fig. 2, the HA1 amino acid sequence is numbered starting from this Asp residue. The HA2 amino terminus would begin at the Gly-1 residue following Arg-325, which constitutes the cleavage site. The hydrophobic membrane-spanning domain near the carboxy terminus of HA2 lies approximately between Ala-187 and Ser-213. The mature hemagglutinin therefore consists of HA1 of 324 amino acids (323 in AS H13) and HA2 of 223 amino acids. There is a single arginine at the cleavage site between HA1 and HA2 in each of the three HAs. Thus the cleavage site of H13 HA resembles those of mammalian and avirulent avian influenza viruses, whereas the highly virulent avian influenza viruses possess multiple arginine or lysine residues at the cleavage site (Kawaoka *et al.*, 1987).

The 3 HA proteins share potential N-linked carbohydrate attachment sites (Asn–X–Ser/Thr) at residues 11, 164, 165, and 287 in HA1 and 145 and 154 in HA2. The MD and WH HAs have an additional site at HA1 residue 36, while the AS HA has an additional site at HA2 residue 158. These positions are similar to those found in other HA subtypes. We have not determined to which of these sites carbohydrate is actually attached.

Comparison of H13 HA with other HA subtypes

Amino acid homologies between H13 (MD) HA and the other HA subtypes are shown in Table 2. The comparisons shown for the subtypes H6, H8, H9, H11, and H12 are similar to those of Hinshaw *et al.* (1982), since no new sequence information is available for these subtypes. Hinshaw *et al.* (1982) have previously identified H11 as the subtype most closely resembling H13 (62% homology in HA1 amino terminus). The H13 sub-

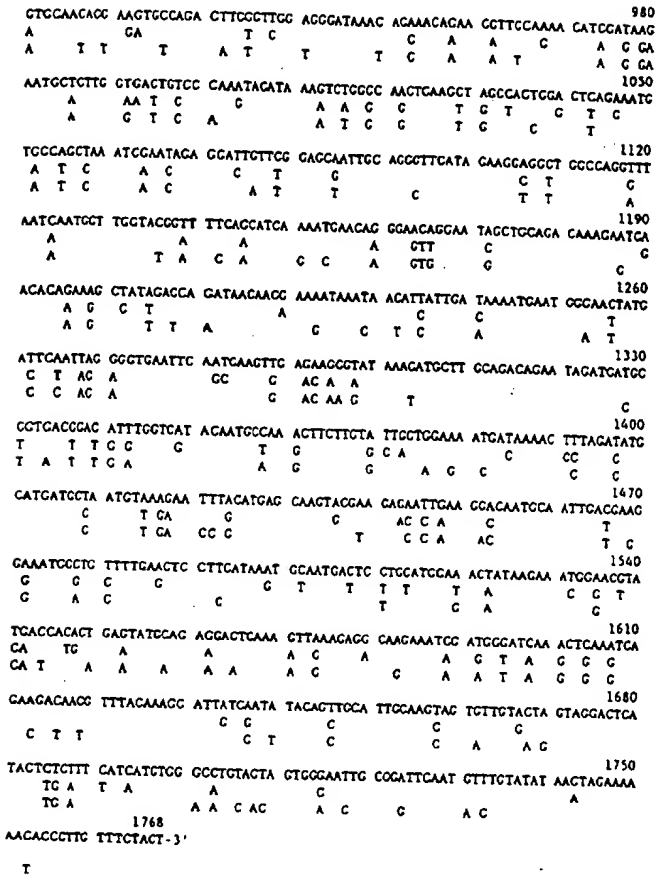

FIG. 1—*Continued*

type is clearly distinct from all other HA subtypes for which complete sequences are available. As has been previously observed among other HA subtypes (Ward, 1981), H13 HA resembles other subtypes more closely in HA2 than in HA1, with about 50% homology to each. Cysteine residues in H13 HA can be aligned with those in other subtypes, except that the cysteine at −5 from the carboxy terminus of HA2 in other subtypes is displaced to −7 in H13 HA. Additionally, 10 of 16 proline residues in H13 (MD) HA1 can be aligned with prolines of most other HA subtypes. Maximum alignment among the different subtypes requires the introduction of small gaps. We have aligned MD H13 HA with Aichi H3 HA by introducing in the H13 sequence one-residue gaps following residues 26, 34, and 108, and an eight-residue gap following residue 40; and in the H3 sequence one-residue gaps following residues 52, 76, 93, 146, and 255 in HA1 and 180 and 189 in HA2, and two-residue gaps following 123 in HA1 and in the signal sequence. Alignment of H13 HA with other subtypes such as H1 is more simple.

Table 2 also shows the extent of H13 HA similarity to type B and C influenza HAs. H13/B homology is comparable to reported values for type B and other type A HAs such as PR/8/34 H1 HA (Krystal *et al.*, 1982). Type C HA is much different from H13 and other HAs as described previously (Nakada *et al.*, 1984).

Examination of the H13 sequence for conserved residues involved in receptor binding reveals the following: nearly all amino acids identified in H3 HA as forming the receptor-binding cavity and "second shell" (H3 numbering: Tyr-98, Gly-134, Ser/Thr-136, Ala-138, Phe-147, Phe/Tyr-148, Trp-153, His-183, Glu-190, Leu-194, Tyr-195, Arg/Asn-224, Gly-225) (Weis *et al.*, 1988; Wiley and Skehel, 1987) are conserved in H13 HA as well as in all other completely sequenced HA subtypes. Additionally, H13 HA possesses Gln-226 in common with other subtypes except for human and

TABLE 1

PERCENTAGE NUCLEOTIDE AND AMINO ACID SEQUENCE HOMOLOGIES AMONG H13 HAs*

HA	Percentage nucleotide sequence homologies MD	WH	AS
MD		80.8	78.1
WH	89.5 (HA1) 94.6 (HA2)		85.0
AS	83.4 91.5	88.9 93.3	

Percentage amino acid sequence homologies

* Percentage nucleotide sequence homologies are above the diagonal. Percentage amino acid homologies are below the diagonal. These are partitioned into HA1 and HA2 polypeptides and exclude the amino-terminal signal sequence.

some swine H3 viruses. In H3, Gln-226 is associated with resistance to hemagglutination inhibition by the α2 macroglobulin of horse serum, whereas Leu-226 confers sensitivity to horse serum (Rogers *et al.*, 1983). In an HI assay, the three H13 viruses were each horse serum resistant (data not shown).

However, H13 differs from other subtypes at positions 227 and 229. H13 has Arg/Lys-227, whereas other sequenced avian influenza virus HAs have Ser-227 (except Duck/Ukraine/63, Pro-227). Swine, equine, and human HAs have serine, alanine, glycine, or glutamic acid at this position (Both *et al.*, 1983; Kawaoka *et al.*, 1989, Kida *et al.*, 1988). Also H13 has Trp-229, whereas nearly all other HAs have Arg-229. The only known exceptions to this are a single human H3 strain, a duck H3 strain, and a seal H4 strain which have Gly-229 (Both *et al.*, 1983; Kida *et al.*, 1987; Donis *et al.*, 1989). Since positions 227 and 229 have not been implicated in receptor binding but are relatively highly conserved among other subtypes, the significance of the violation of this conservation in the H13 HA is not known.

Antigenic analysis of H13 viruses

A panel of monoclonal antibodies was prepared against the hemagglutinin of AS virus. These antibodies did not react in HI tests with H1, H2, or H3 viruses, although we have not tested for cross-reactivity with other HA subtypes. The epitopes on AS HA with which the antibodies react have not been determined. We employed this panel to estimate the degree of antigenic similarity of H13 influenza viruses distributed geographically and over time (Table 3). A total of 27 viruses were examined, including 11 isolated from waterfowl in the Caspian Sea region of the USSR, and 16 from the USA, predominantly from its eastern seaboard. These viruses were isolated from cloacal swabs or feces of birds (except for the whale isolate) and grown in embryonated chicken eggs. Each virus was characterized as H13 based on HI reactivity with polyvalent rabbit serum raised against MD virus (Hinshaw *et al.*, 1982). As shown in Table 3, we found wide variability in reactivity patterns. Considered as groups, the reactivity patterns of H13 viruses from the USSR and USA were dissimilar. Seven of eleven USSR viruses, but only 1/16 USA viruses, reacted with more than half of the panel of antibodies. Also, 6/16 USA viruses, but no USSR viruses, did not react with any monoclonal antibody. The majority of USSR H13 viruses reacted

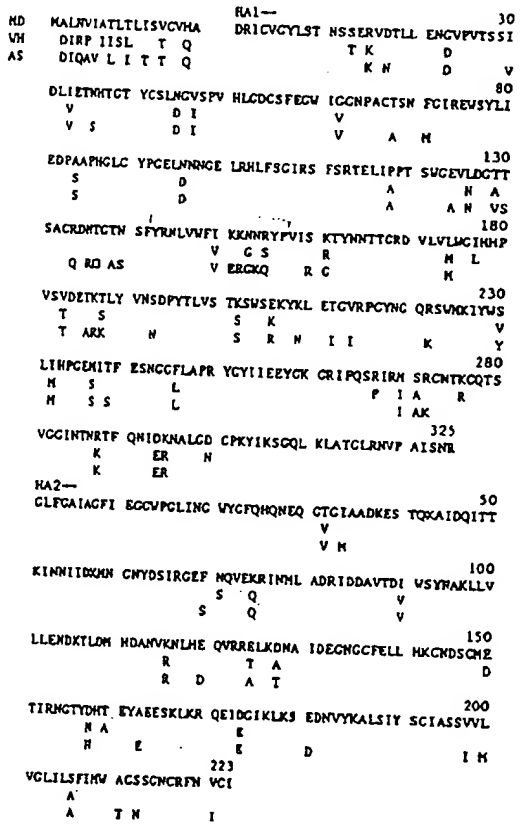

FIG. 2. Amino acid sequences of H13 HA proteins. The MD sequence is shown in its entirety on the top line, while amino acids in the WH and AS HA proteins which differ from MD HA are shown underneath. The closed box in the AS sequence at position 137 (or, alternatively, 136) of HA1 indicates that no counterpart of this position in MD HA exists in AS HA. The probable NH_2 termini of HA1 and HA2 in the mature protein are indicated in the figure.

TABLE 2

H13 AMINO ACID HOMOLOGY WITH OTHER VIRUS SUBTYPES (%)

Subtype	Strain	HA1	HA2	Reference
H1	PR/8/34	41	56	Winter *et al.*, 1981
H2	Japan/305/57	42	53	Gething *et al.*, 1980
H3	Aichi/2/68	30	52	Verhoeyen *et al.*, 1980
H4	Ruddy Turnstone/NJ/47/85	32	54	Donis *et al.*, 1989
H5	Turkey/Ireland/1378/83	45	57	Kawaoka *et al.*, 1987
H7	Seal/MA/1/80	31	49	Naeve and Webster, 1983
H10	Mink/Sweden/84	32	50	Feldmann *et al.*, 1988
H6	Shearwater/Australia/72	31	—*	Air, 1981
H8	Turkey/Ontario/6118/68	43	—	Air, 1981
H9	Turkey/WS/1/66	48	—	Air, 1981
H11	Tern/Australia/75	62	—	Air, 1981
H12	Duck/Alberta/60/76	44	—	Air, 1981
Type B HA	B/Lee/40	26	33	Krystal *et al.*, 1982
Type C HA	C/CA/78	25	19	Nakada *et al.*, 1984

* No data, complete sequences are not available. HA1 homology value is for amino terminus only (67–82 residues); signal peptide is not included.

more weakly with polyvalent serum against MD virus than did most USA viruses. This can be interpreted as reflecting the divergent evolution of viruses enforced by the partial geographical isolation, between the eastern and western hemispheres, of shorebird migration paths. A similar argument has been made for avian H4 viruses (Donis *et al.*, 1989). There is no indication in these data of a linear evolutionary trend; in both the USSR and the USA, viruses with HA antigenically similar to MD virus were isolated in 1986 together with antigenic relatives of AS virus. Among the USSR viruses, but not the USA viruses, there is a pattern of appearance during the early 1980s of AS HA-like epitopes recognized by monoclonal antibodies 1, 2, 6, 9, and 32. The USSR viruses from the mid-1970s react similarly to MD (1977) virus against the monoclonal antibody panel, although not as strongly against polyvalent serum.

DISCUSSION

The existence in natural avian reservoirs of HA subtypes of influenza virus differing in both antigenic and functional characteristics from those now circulating in humans, and their evident ability to infect mammalian species, makes necessary an understanding of the features of these HA subtypes that determine their host range and virulence. We have determined the nucleotide sequences of subtype H13 HA genes as a first step toward answering two questions: (1) What features of the H13 HA are involved in restriction of virus replication in ducks? (2) What is the potential of H13 viruses to infect other species including humans?

Some functional determinants are similar in H13 HA and other HA subtypes. For example, the amino terminus of HA2, which mediates fusion between viral and cellular membranes, is common among H13 and all other completely sequenced HA subtypes in 13 of the first 14 amino acids (Lamb, 1983). The cleavage site between HA1 and HA2 consists of a single arginine, as in other subtypes except for some high-virulence strains of the H5 and H7 subtypes (Kawaoka *et al.*, 1987). At the receptor binding site, all the amino acids identified as being directly involved in receptor binding in H3 HA are conserved in H13 HA as well as in other subtypes (Weis *et al.*, 1988; Wiley and Skehel, 1987). As with HAs from other avian influenza viruses, H13 HAs have glutamine at position 226 (H3 numbering) and correspondingly are resistant to hemagglutination inhibition by horse serum (Rogers *et al.*, 1983).

However, changes in amino acid residues adjacent to the receptor binding site can influence receptor specificity (Daniels *et al.*, 1987). H13 HA is unique among sequenced HA subtypes by having arginine or lysine at residue 227 and tryptophan at 229 (H3 numbering). The effect of these substitutions on the receptor binding properties of H13 HA are unknown. H13 HA also has Ser-228, which is very rare among HAs from avian influenza isolates. Naeve *et al.* (1984) have demonstrated that in H3 HA containing Gln-226 and Ser-227, substitution of Ser-228 for Gly-228 was associated with loss of virus replicative ability in the intestinal tract of ducks, even after rectal inoculation. Thus it is possible that this single substitution may be responsible for the absence of H13 viruses in the wild duck res-

TABLE 3

HI REACTIVITY OF ANTI-HA MONOCLONAL ANTIBODIES WITH DIFFERENT H13 VIRUSES

Origin	Virus	Polyclonal serum[b]	Monoclonal antibodies[a] 1	2	4	5	6	9	20	32
USSR	Gull/Astrachan/227/84 (AS)[c]	160	6,400[d]	3200	400	100	12,800	12,800	800	6400
	Gull/Astrachan/28/76[e]	64	<	<	200	<	<	<	800	<
	Gull/Turkmenia/13/77	256	<	<	400	<	<	<	<	<
	Gull/Astrachan/1421/79	64	400	<	400	<	<	800	800	<
	Gull/Astrachan/591/82	2048	3,200	800	<	<	<	12,800	1600	6400
	Gull/Astrachan/75/83	512	1,600	<	200	100	<	12,800	<	3200
USA	Gull/MD/704/77 (MD)[f]	640	<	<	200	<	<	<	1600	<
	Whale/ME/328HN/84 (WH)	1280	<	<	200	<	<	<	800	<
	Gull/MD/1824/78[g]	640	<	<	<	<	<	<	<	<
	Shorebird/NJ/840/86	320	12,800	800	400	<	800	800	<	<
	Gull/ME/16/85	1280	<	<	100	<	<	<	<	<
	Gull/DE/2635/87	2560	<	200	<	<	<	<	<	<

[a] Monoclonal antibodies were raised against the HA of AS virus.

[b] Polyclonal rabbit serum was raised against the HA of MD virus (Hinshaw *et al.*, 1982).

[c] Similar reactivity patterns were found with the viruses Gull/Astrachan/458/85, Gull/Astrachan/165/86, Gull/Astrachan/176/88, and Gull/Astrachan/178/87.

[d] HI titers represent the reciprocal of the highest dilution of monoclonal antibodies (up to 1:12,800) inhibiting 4 HA units of virus. < represents a titer of less than 100.

[e] A similar pattern was found with Teal/Volga/671/86 virus.

[f] Similar patterns were found with viruses Gull/MD/4574/79, Gull/MN/945/80, Gull/MN/1352/81, Gull/DE/1231/86, and Gull/DE/2592/87.

[g] Similar patterns were found with viruses Gull/MD/5393/80, Gull/DE/439/86, Gull/DE/990/86, Gull/DE/1370/86, and Gull/DE/660/88.

ervoir. However, Naeve *et al.* (1984) leave open the possibility that other substitutions might have affected their mutant's phenotype. The whale H13 virus, which also has Ser-228, replicates in the duck intestinal tract if inoculated rectally although not if inoculated orally (Hinshaw *et al.*, 1986). This suggests that restriction of H13 virus replication in ducks is not due to a failure to bind to duck cellular receptors. This restriction might instead be due to increased acid lability of H13 viruses compared to duck viruses. H13 viruses might be capable of surviving transit through the digestive tract of gulls, but not of ducks. If so, increased acid lability should be reflected in a higher pH threshold for the HA conformational change required for fusion to endosomal membranes. Substitutions in HA near the receptor-binding site can alter this pH threshold (Daniels *et al.*, 1985, 1987). However, in a hemolysis assay for HA conformational change, we found that the 50% hemolysis pH for MD and AS viruses (which do not replicate in ducks) and for Duck/Alberta/92/76 virus were very similar at about 5.5 (T. Chambers and R. Webster, unpublished data). Thus the relevance of specific residues in H13 HA to the restriction of H13 virus replication in ducks remains to be demonstrated.

The isolation of H13 viruses from a diseased whale and the ability of MD and WH viruses to replicate in ferrets clearly indicate that such viruses have the ability to infect mammals. However, it is not known whether the WH virus was the cause of disease in that whale, or an opportunistic infection. There is no evidence indicating that H13 viruses were transmitted from whale to whale, although mass stranding and death of pilot whales was going on at that time. Epizootics of influenza in marine mammals have occurred, such as the high mortality outbreaks in seals of H7 influenza in 1979–80 and H4 influenza in 1982–83 (Geraci *et al.*, 1982; Hinshaw *et al.*, 1984). Other influenza viruses have been detected in whales (Lvov *et al.*, 1978). Unlike influenza in humans or horses, however, there is no evidence that influenza viruses are permanently maintained in marine mammals. The sequences of the HAs of the 1980 and 1982 seal virus isolates, like the whale H13 HA, resemble avian HAs of the same subtypes (Naeve and Webster, 1983; Donis *et al.*, 1989). Similarly, based on competitive RNA–RNA hybridization the nucleoprotein (NP) genes of the Seal H4 and H7 viruses and of the whale H13 virus resemble the NP genes of avian viruses of the same HA subtype, but not the NP genes of swine, equine, or human influenza viruses (Bean, 1984; Hinshaw *et al.*, 1986). Probably, influenza epizootics in marine mammals were each caused by the introduction of avian influenza virus di-

rectly from an avian source. The failure of these viruses to maintain themselves permanently in the new hosts could be due to low average population density, absence of a confining environment, or perhaps a requirement for special circumstances to facilitate infection, such as seasonal host clustering behavior, adverse weather, or coincidental infection with bacteria (Tashiro *et al.*, 1987) or mycoplasma (Geraci *et al.*, 1982).

Adaptation of avian influenza viruses to mammals may require mutations in HA or in other virus genes. Although none of the substitutions in WH HA can yet be clearly correlated with host adaptation, one area that might be involved is at HA1 residues 151 and 153 (156, 158 by H3 numbering), located in the globular head of the molecule in the vicinity of the receptor binding site. The WH HA, but not the gull H13 HAs, is identical at both positions to the sequence of the L-phenotype H1 swine viruses, which exhibit enhanced replication in swine and diminished replication in embryonated chicken eggs. In H1 viruses the L/H host adaptation phenotype is specifically determined by these two positions in HA (Kilbourne *et al.*, 1988). It is possible that the equivalent positions in other subtypes are also involved in host adaptation.

Antigenic analysis of 28 H13 viruses using a panel of monoclonal antibodies gave no indication of a single linear evolutionary trend. Although not proven, it seems likely on this basis that H13 viruses have been evolving in multiple independent lineages, as has been suggested for avian H3 and H4 HA genes (Kida *et al.*, 1987; Donis *et al.*, 1989). H13 strains from the eastern and western hemispheres appear generally to be diverging from each other. However, Kawaoka *et al.* (1989) have found that antigenic analysis and nucleotide sequence analysis of evolutionary patterns can give conflicting results. In their examination of the evolution of H3 HA genes of equine influenza viruses, antigenic analysis suggested that multiple strains were cocirculating, as we observed here; whereas the nucleotide sequence analysis of the same set of genes indicated HA evolution in a single lineage. Therefore, without more extensive nucleotide sequence information we cannot make a clear judgement of the evolutionary patterns of H13 HA genes.

ACKNOWLEDGMENTS

This work was supported by USPHS Contract No. AI-52586 from the National Institutes of Health, CORE Grant CA 21765, and American Lebanese Syrian Associated Charities. We thank John Freeman and Daniel Channell for technical assistance, and Dayna Anderson for typing the manuscript. The nucleotide and deduced amino acid sequences have been submitted to Genbank.

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